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Toxicology Study No. S.0043494c-16, November 2017
Toxicology Directorate

Human Cell Line Activation Test of the Novel Energetics methyl trinitropryrazol (MTNP) and 1,3-dimethylhexahydropyrimidine (DHP)

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14. ABSTRACT
The energetic and toxicological properties of methyl trinitropyrazol (MTNP) and 1,3-dimethylhexahydropyrimidine (DHP) are being determined to support an evaluation of these compounds as replacements for energetics in current use, such as such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and trinitrotoluene (TNT). This study assessed the skin sensitization potential of MTNP and DHP through the human cell line activation test (h-CLAT), an in vitro approach to assess dendritic cell activation by chemicals, a critical step in the induction of a skin allergy. MTNP induced a positive reaction in the assay, while DHP did not.

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Good Laboratory Practice Compliance Statement

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:

1. The test article characterization (purity) was conducted by the manufacturer and it is not known whether the testing was done in compliance with the above regulation.

2. Due to time constraints, the method of analysis for these compounds could not be validated by the Laboratory Sciences Portfolio (LAB) prior to the study start in compliance with GLP requirements. Because of this the dosing solutions used for all tests are frozen (at - 80 degrees C) until the method can be validated by the LAB after study completion.

3. Due to calibration error, the balance used for verifying pipette function was flagged as "in need of repair". Four years of weight set verification data logs were reviewed and the balance is operable and functioning properly. The balance was continued in use and weight set verification was performed prior to each day's use.

4. At the time the study was conducted, there was not an APHC approved GLP protocol. The method has been validated and a formal report documenting the protocol and local changes has been completed. The current study followed the methodology as described in the formal report and all steps were documented and are found in the archives. No deviations from the aforementioned regulation affected the quality or integrity of the study or the interpretation of the results.

Emily N. Reinke, Ph.D.
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6 November 2017
Date

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1 Summary

1.1 Overview

The energetic and toxicological properties of methyl trinitropyrazol (MTNP) and 1,3-dimethylhexahydropyrimidine (DHP) are being determined to support an evaluation of these compounds as replacements for energetics in current use, such as such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and trinitrotoluene (TNT). The following study assessed the skin sensitization potential of MTNP and DHP through the human cell line activation test (h-CLAT), an *in vitro* approach to assess activation of dendritic cells, a critical step in the elicitation of a sensitizing response. Data from this study are used to assist in making environment and health-based decisions regarding the design and selection of formulas and materials for further development of new munition compounds.

1.2 Purpose

The purpose of this study is to provide environmental and occupational health information on new or replacement energetic compounds for military use. This information is critical to the research, development, testing, and evaluation (RDT&E) of munition formulation alternatives. This study addresses, in part, the environmental safety and occupational health (ESOH) requirements outlined in Department of the Army (DA) Regulation 200-1[1]; DA Regulation 40-5 [2]; and DA Regulation 70-1 [3]; Department of Defense Instruction 4715.4 [4]; and Army Environmental Research and Technology Assessment (AERTA requirement PP-3-02-05 [5], Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces. This program is under the direction of the U.S. Army Research, Development and Engineering Command (USARDECOM) Environmental Technology Acquisition Program and Environmental Quality Technology (EQT) Pollution Prevention.

Research, development, testing, training, and use of substances potentially less hazardous to human health and the environment is vital to the readiness of the U.S. military. Safeguarding the health of Soldiers, civilians, and the environment requires an assessment of alternatives before they are fielded. Continuous assessments begun early in the RDT&E process can save significant time and effort during RDT&E, as well as over the life cycle of the items developed. Residues of pyrotechnics, propellants, explosives, and incendiaries have been found in soil, air, surface, and groundwater samples, creating environmental problems and interfering with training activities.

The Department of the Army is identifying replacements for substances causing environmental and/or occupational risks to health. The purpose of this toxicology study was to examine the skin sensitization potential of MTNP and DHP using the h-CLAT assay, and to conduct the assay consistent with Good Laboratory Practice (GLP) Standard Regulations.

1.3 Conclusions

MTNP was found to elicit a positive reaction for both sensitization markers in the THP-1 monocytic leukemia cell line, a dendritic cell surrogate. Both CD54 and CD86 expression levels were

increased as a result of 24-hour exposure to MTNP. DHP was not found to elicit a reaction in the THP-1 cell line. Thus, only MTNP is considered a skin sensitizer according to the h-CLAT test.

1.4 Recommendations

MTNP appears to be a skin sensitizer upon analysis with the h-CLAT skin sensitizing assay when combined with Quantitative Structure-Activity Relationship (QSAR) analysis [6]. DHP is considered to be negative for skin sensitization by this assay; however, QSAR analysis suggested that it might be a skin sensitizer. Further *in vitro* or *in vivo* testing is recommended to more definitively determine the sensitizing potential of these compounds. The h-CLAT is one of many non-animal skin sensitizing tests, and it comprises part of an integrated testing strategy with two other *in vitro* approaches, the DPRA and the LuSens assay [7-11]. A comprehensive assessment of skin sensitization potential requires results from all three assays along with specific *in silico* analysis provides a more robust estimation of skin sensitization than h-CLAT alone [12]. As testing has only occurred with the h-CLAT, it is not yet possible to provide a definitive response as to the sensitization potential. Testing with the direct peptide reactivity assay will be conducted, and the LuSens test is currently under validation by APHC and should be available to complete the *in vitro* tests necessary for analysis. However, the robustness of the h-CLAT assay provides a strong indication that MTNP is a skin sensitizer and appropriate precautions should be taken when handling the material.

2 References

See Appendix A for list of references

3 Authority

Military Interdepartmental Purchase Request No. 10896394. This technical report addresses, in part, the environment, safety and occupational health (ESOH) requirements outlined in Department of Defense Instruction (DODI) 4715.4 [13], Department of the Army Regulation (AR) 200-1, Environmental Protection and Enhancement[14]; AR 40-5, Preventive Medicine [15]; and AR 70-1, Army Acquisition Policy [16]; Department of Defense Instruction 4715.4, Pollution Prevention [13]; and Army Environmental Research and Technology Assessment Requirement PP-3-02-05, Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces . It was conducted as part of an on-going effort by the U.S. Army Research, Development and Engineering Command (RDECOM), Environmental Technology Acquisition Program (ETAP, Mr. Erik Hangeland) and the Environmental Quality Technology (EQT) Pollution Prevention Team (P2TT), co-chaired by Dr. John LaScala.

4 Background

Current regulations require the assessment of human health and environmental effects arising from exposure to substances in soil, surface water, and groundwater. Applied after an item has been fielded, these assessments can reveal the existence of adverse environmental and human health effects that must be addressed, often at substantial cost. It is more efficient to begin the assessment of exposure, effects, and environmental transport of military-related compounds/ substances early in the RDT&E process to avoid unnecessary costs, conserve physical resources, and sustain the health of those potentially exposed. The U.S. Army RDECOM, ETAP has been dedicated to finding replacements for substances known to cause environmental and/or occupational risks to health or developing less hazardous new explosives. A goal of this program is

to investigate these new compounds with operational and/or environment, safety, and occupational health issues. The candidates under development for high density energetics include TNBA and DHP.

National defense requires the development of unique energetic compounds to perform specialized mission requirements. These requirements also include the sustainable use of these materials in the environment, particularly during training operations. The use of RDX (1,3,5-hexahydro-1,3,5-trinitrotriazine) and trinitrotoluene (TNT) in warheads has constrained use of training ranges potentially affecting military readiness. Unexploded ordnance and low-order detonations have become sources of ground water contamination and have affected drinking water resources.

The Centers for Disease Control and Prevention (CDC), Agency for Toxic Substances and Disease Registry (ATSDR) has developed an acute oral minimum risk level (MRL) for RDX of 60 micrograms per kilograms per day (µg/kg-day) based on its epileptiform seizure neurotoxicity in humans and rodents [17-20]. The USEPA has derived a chronic reference dose (RfD) of 3 µg/kg-day based prostatic inflammation in rodents. RDX is also classified as a possible carcinogen [21, 22].

TNT is acutely toxic to rats causing ataxia, tremors, and mild convulsions; oral LD_{50} values range from 660 to 1320 mg/kg. The reference dose (RfD) for subchronic and chronic oral exposures of 0.0005 mg/kg-day is based on a LOAEL of 0.5 mg/kg-day for liver effects in dogs. TNT is classified in weight-of-evidence Group C, possible human carcinogen [23, 24].

The Army Technology Acquisition Program (ETAP) is dedicated to finding replacements for RDX and TNT that will reduce or eliminate the health risks from environmental exposure and will reduce adverse ESOH effects; RDX adversely affects the readiness and costs associated with training [25]. To support the development of sustainable, low-toxicity materials for use, fast, high-throughput methods are needed to assess relative toxicity of new munition compounds as they are developed. Toxicity tests can be conducted *in vivo* and *in vitro*. *In vitro* methods have the advantage of being relatively inexpensive, high-throughput, and capable of addressing many mechanistic issues at the cellular and molecular level. Specifically for the newly developed materials, the *in vitro* tests are most suitable and effective screening tools, given that often very limited amounts of test substances are available. By identifying ESOH effects early in the acquisition process, unacceptable replacement compounds can be identified.

The Army Public Health Center, Toxicology Directorate (APHC TOX) was tasked with providing acute toxicity data for DHP and MTNP to determine their potential environmental and occupational hazards, which includes skin sensitization. The data from these studies will help in making recommendations for continued development and toxicity testing resulting in appropriate exposure guidance.

MTNP (Chemical Abstract Number (CASRN) unknown) and DHP (CASRN unknown) are novel energetics under evaluation as replacements for RDX and TNT. Few toxicity data on the compound exist, however, QSAR analysis indicates that both compounds may be strong sensitizers [6]. The Toxicology Directorate of the Army Public Health Center has been tasked with evaluating the skin sensitization potential of MTNP and DHP. Testing in the h-CLAT *in vitro* system constitutes the first evaluation of these compounds *in vitro* skin sensitization methods; multiple test systems are required to confirm results.

The h-CLAT is an *in vitro* approach to analyze dendritic cell activation of test chemicals via the expression of CD54 and CD86 on the cell surface. There are several key steps in the elicitation of a skin sensitizing reaction, including the activation of dendritic cells and the transformation from antigen processing to antigen presenting cells [26]. Multiple cell surface markers are expressed by dendritic cells, with CD54 and CD86 being just two examples. The increase in expression on the cellular surface of these proteins is measured by flow cytometry as a result of a fluorescent signal on the antibodies which bind to either CD54 or CD86 [27-29]. The criteria for a positive reaction in h-CLAT require either a 2-fold or a 1.5-fold induction of CD54 or CD86 respectively as compared to solvent controls. During a skin sensitizing reaction, activated dendritic cells migrate to the lymph node where the major histocompatibility complexes which they are presenting activated T-cells and T-cell proliferation. Secondary exposure to the chemical will then result in inflammation and an allergic reaction.

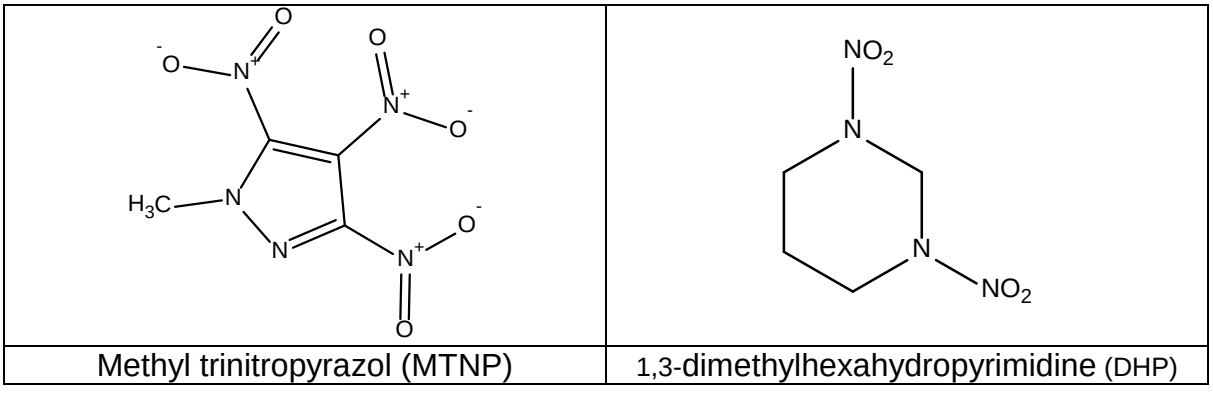
5 Materials and Methods

5.1 Materials

5.1.1 Test Substance

Synthesis of MTNP (CASRN unregistered) was completed by the Holston Army Ammunition Plant, Kingsport, TN. DHP (CASRN unregistered) was synthesized by Dr. Joseph Banning of the Army Research Lab, Aberdeen Proving Ground, MD. Full purity analyses for these compounds were not provided by the sponsor, however correspondence with the sponsor has indicated that purity is > 99 percent. The molecular structures of the compounds are shown in Figure 1.

Both compounds were fully soluble at 500 mg/mL in DMSO.



Methyl trinitropyrazol (MTNP)	1,3-dimethylhexahydropyrimidine (DHP)

Figure 1. Molecular Structure of the Compounds

5.1.2 Cell Line, Chemicals and Reagents

The h-CLAT has undergone validation testing within the APHC to verify that the assay performs as expected with APHC equipment when compared to published results [30]. THP-1 cells were

acquired from the American Type Tissue Collection (ATCC, Manassas, VA). All tissue culture reagents were acquired from Gibco, a subsidiary of ThermoFisher (Waltham, MA). Cells were cultured in RPMI-1640 containing 10 percent fetal bovine serum, 100 U/mL penicillin, 10 µg/mL streptomycin and 0.05 mM 2-mercaptoethanol. Reagents for flow cytometry were as follows: physiological saline (Sigma-Aldrich, Inc., St. Louis, MO), dimethyl sulfoxide (DMSO, Sigma-Aldrich, Inc.), Dulbecco's phosphate buffered saline without calcium, magnesium or phenol red (Gibco, Inc.), bovine serum albumin fraction V (BSA, Calbiochem, Billerica, MA), globulins Cohn fraction II, II, human (Sigma-Aldrich, Inc.), and propidium iodide (PI, Sigma-Aldrich, Inc.). Control test chemicals were all obtained from Sigma-Aldrich, Inc., to include 2,4-dinitochlorobenzene (DNCB, CASRN 97-00-7), nickel sulfate ($NiSO_4$, CASRN 7786-81-4), and lactic acid (LA, CASRN 50-21-5). Antibodies against IgG1 (control) and CD54 were obtained from Dako (Carpinteria, CA) and antibodies against CD86 (Clone 2331, Fun-1) were obtained from BD Biosciences (San Jose, CA). All antibodies were tagged with the FITC fluorophore. All cells, reagents and chemicals were stored according to manufacturer's instructions. Lot numbers and expirations dates for all reagents used are provided in Appendix D, certificates of analysis are available at the individual manufacturer's websites.

5.1.3 Equipment

The assay reaction was analyzed by flow cytometry utilizing a BD FACSVerse flow cytometer (BD Biosciences)[31] .

5.1.4 Quality Assurance

Army Public Health Center policy requires that all experiments and studies conducted by any element of the APHC Directorate of Toxicology will be compliant with the applicable Good Laboratory Practice (GLP) Standard guideline [32]. For this study, the test article dictates that the following GLP guideline applies [33]:

Code of Federal Regulations (CFR), Title 40: Protection of Environment, Part 792-Good Laboratory Practice Standards.

According to this policy and that these results may be used in regulatory decisions involving the EPA, these Ames test assays were conducted in compliance with GLP standards and followed the appropriate regulatory testing guidelines [34].

In compliance with the GLP requirements, the PHC Quality Systems Office audited critical phases of this study. A Quality Assurance Statement is provided in Appendix B, which provides the dates of these audits along with the audited phases and the dates that the results of the audits were reported to Management and the Study Director. The additional Quality Assurance/GLP requirement of archives location is provided in Appendix C as well as the names of personnel contributing to the performance of this study.

No certificates of analysis from the manufacturers were received for the test compounds.

5.2 Methods

All assay setup was performed according to ECVAM DB-ALM protocol number 158, OECD Guideline [28, 29].

5.2.1 Buffers

FACS buffer was prepared with PBS and 0.1 percent (w/v) BSA the day before use and stored at +4 ± 2 ^{O}C. Blocking solution was made up in 1 percent (w/v) globulins in PBS stocks as needed, with stock being used within one week and stored at +4 ^{O}C. Blocking solution for use on the day of the experiment was diluted to a 0.1 percent solution in FACS buffer immediately prior to use. PI was diluted to 12.5 µg/mL in PBS on the day of the experiment and maintained on ice.

5.2.2 Tissue Culture

Tissue culture media was prepared as described in section 5.1.2 and maintained at +4 ± 2 ^{O}C. Media was pre-warmed at room temperature prior to use for each cell plating and passage. Cells were maintained at 1.5 x 10^5 – 8 x 10^5 cells/mL and were routinely passaged every 2-3 days. Cells were maintained at 37 ^{O}C, 5 percent CO_2. Cells for the assay were in culture for no more than 30 passages or 60 days. Prior to passage or test plating, cell density was determined by counting with the TC-20 automated cell counter (Bio-Rad, Inc., Hercules, CA). Cell viability was determined by Trypan blue staining (Bio-Rad, Inc.). For range finding and h-CLAT testing, cells were plated into 24-well plates at a density of 1 x 10^6 cells/well in 0.5 mL. For maintenance, cells were plated at 1.5-2.0 x 10^5 cells/mL in 25-40 mL media depending on the timing of subsequent tests.

5.2.3 Reactivity Check

Two weeks after cells were thawed; a reactivity check on the batch is carried out utilizing DNCB, $NiSO_4$ and LA. Each flask of cells was tested separately. DNCB was prepared in 20 mg/mL stock solutions in DMSO and diluted to 2.4 mg/mL in DMSO. Stock solutions of DNCB were maintained at +4 ^{O}C. Serial dilutions of 1:1.2 were carried out for a total of 2 dosing levels and subsequently diluted 1:250 in 0.5 mL media. $NiSO_4$ was prepared in a 10 mg/mL solution in saline and diluted 1:50 into 0.5 mL media and 1:34.5 into 0.5 mL media. LA was prepared as a 100 mg/mL solution in saline and diluted 1:50 and 1:34.5 into 0.5 mL media. One 1:1.2 dilution was made. DNCB, $NiSO_4$ and LA were then diluted 1:2 into the 0.5 mL containing 1 x 10^6 cells. A dead cell well was prepared by diluting 10 µL of 20 mg/mL DNCB (final concentration 0.2 mg/mL) into 1 mL of media containing 1 x 10^6 cells in a 24-well plate. DMSO and saline control wells were also prepared. The plate was incubated for 24 hours and cells and were processed and stained for IgG1, CD54 and CD86 and analyzed by flow cytometry (section 5.2.6).

5.2.4 Range finding

In order to determine appropriate dosing levels, TNBA and DHP were initially prepared in a 500 mg/mL solution in DMSO. Initial range finding concentrations were based on solubility at the maximum concentration recommended by the protocol. Serial dilutions (1:2) were prepared in DMSO for a total of 8 dilutions. Each dilution was further diluted 1:250 into 0.5 mL media and diluted 1:2 into 0.5 mL media containing 1 x 10^6 cells in a 24-well plate. Cells from individual flasks were combined prior to plating for the assay. As described in 5.2.3, a DMSO control and dead cell control were prepared. Cells were incubated for 24 hours. Following incubation, each sample was transferred to a 5 mL tube and centrifuged at 200 x g at +4 ^{O}C. Pellets were resuspended in 0.6 mL cold FACS buffer and 0.2 mL transferred to flow cytometry sample tubes. Samples were

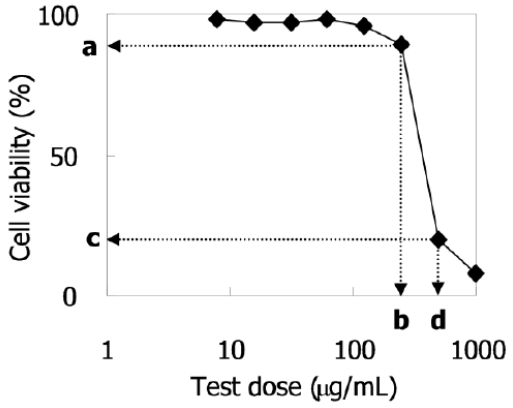
washed twice in 0.2 mL FACS buffer, resuspended in 0.4 mL FACS buffer and stained with 20 µL of a 12.5 µg/mL PI stock (final concentration 0.625 µg/mL). Samples were maintained on ice in the dark and assayed for viability by flow cytometry. The dead cell control and the saline control were used to gate out dead cells stained with PI and the flow cytometer was set to acquire 10,000 live cell hits (PI negative) or 30,000 total hits, whichever was achieved first. Percent viability (ratio of live cells to total acquired cells) was utilized to determine the 75 percent cell viability (CV75) by the following equation (see also Figure 2):

$$\log CV75 = \frac{(75-c) \times \log b - (75 - a) \times \log d}{a - c}$$

where:
a = Percent viability above 75 percent (nearest dose)
b = Dose level of a
c = Percent viability below 75 percent (nearest dose)
d = Dose level of c
See Figure 1.

The CV75 is the value at which the second highest dose is set for the final test. Additional range finding assays had to be completed for TNBA as it was highly toxic at the initial testing doses.

Figure 2- Example results range finding PI assay*



*ECVAM DB-ALM, *human Cell Line Activation Test (h-CLAT)*, DB-ALM Protocol No. 158. 2015: European Union Reference Laboratory for Alternatives to Animal Testing [28, 29].

The range finding assay was completed a minimum of two times to verify results, if results were similar after two tests, no more testing was completed.

5.2.5 h-CLAT Test

Once the CV75 was determined, a dosing scheme was setup such that the highest dose was 1.2-fold higher than the CV75. MTNP and DHP were weighed and solubilized in DMSO at a

concentration 500x of the 1.2 x CV75. The solution was then diluted in a 1.2 serial dilution for a total of 8 concentration levels and each concentration diluted 1:50 in 0.5 mL complete media. This 0.5 mL was then diluted 1:2 into 0.5 mL containing 1 x 10^6 cells in a 24-well plate. DNCB was prepared from the 20 mg/mL stock by diluting to 2.4 mg/mL in DMSO, serially diluting 1:1.2 for 3 dilutions and then diluting a further 1:250 into media. These were also diluted 1:2 into 0.5 mL media containing 1 x 10^6 cells. A DMSO control was prepared as was a "dead cell" control containing 10 µL of the 20 mg/mL DMSO stock. Cells were incubated for 24 hours and processed for IgG1, CD54 and CD86 staining and analysis by flow cytometry (section 5.2.6).

At the end of the study, the samples were analyzed by the PHC Method Development Section of the -Client Services Division , of the Laboratory Sciences Directorate The final dilution of the empirical starting stock solution for MTNP would theoretically be, for example, 0.318 µg/mL. The validated concentrations of the final serial dilution for MTNP were unavailable at the time this report was written. The expected concentration for DHP dilution was 139.5 mg/mL. The concentrations of the initial concentrates will be back-calculated from these verified concentrations when they are available and an amendment to this report will be provided. The final data will be analyzed from both these concentrations and the nominal concentrations. Where no difference is observed in the final EC150 and EC200 predictions, nominal values will be reported.

5.2.6 Antibody Staining and Flow Cytometry

Each well was transferred containing cells and treatment or treatment control was transferred to a 5 mL snap-cap tube and collected by centrifugation (250 x g, 5 min, +4 ^{O}C) and washed twice in 1 mL cold FACS buffer. Cells were blocked in 0.6 mL 0.1 percent blocking buffer (prepared from the 1 percent stock in FACS buffer) for 15 min. at +4 ± 2 ^{O}C. Following blocking, each sample was split into 3 aliquots of 180-200 µL each in a round-bottom 96-well plate. Samples were spun as above and stained with antibodies. See Table 1 for antibody concentrations.

Table 1 – Antibody concentration

	Volume of antibody	Volume of FACS buffer	Total volume of working solution/sample
FITC labeled-mouse IgG1	6 µL	44 µL	50 µL
Anti-CD54 antibody	3 µL	47 µL	50 µL
Anti-CD86 antibody	3 µL	47 µL	50 µL

A master-mix for each antibody was prepared immediately prior to use and added directly to each cell pellet after removal of the blocking buffer. Each plate was gently vortexed to resuspend the cells in the antibody mix and incubated at +4 ± 2^{O}C in the dark for 30 min. Following the 30-minute incubation, samples were again spun down and washed twice in FACS buffer. Between the first and second wash, samples were transferred to FACS analysis tubes. Samples were maintained on ice throughout the transfer process. Following the final wash, samples were resuspended in 0.4 mL FACS buffer and stained with 20 µL PI. Each sample was gently vortex to mix.

Samples were analyzed by flow cytometry under the following conditions. Acquisition channels were set to read the appropriate acquisition channel for propidium iodide (PI) and fluorescein isothiocyanate (FITC). The following plots were captured for each sample: 2-dimensional plot of forward and side scatter, 2-dimensional dot plot of FITC vs PI and a histogram plot of both FITC and PI. Live cells were used to determine the correct voltages for the forward scatter and side scatter channels. Dead cells were gated out by PI using the dead cell control and the IgG1 saline control and total acquisition was determined by either 10,000 PI negative hits or 30,000 total hits on

the PI channel. For each sample, the geometric mean fluorescence intensity (MFI) was captured for all hits and live/viable cell hits.

From the MFI, the relative fluorescence intensity (RFI) was determined by the following equation:

$$RFI = \frac{\text{MFI of chemical treated cells} - \text{MFI of chemical treated isotype cells}}{\text{MFI of solvent treated cells} - \text{MFI of solvent treated isotype cells}}$$

The cell viability for each concentration was also recorded from the isotype control population.

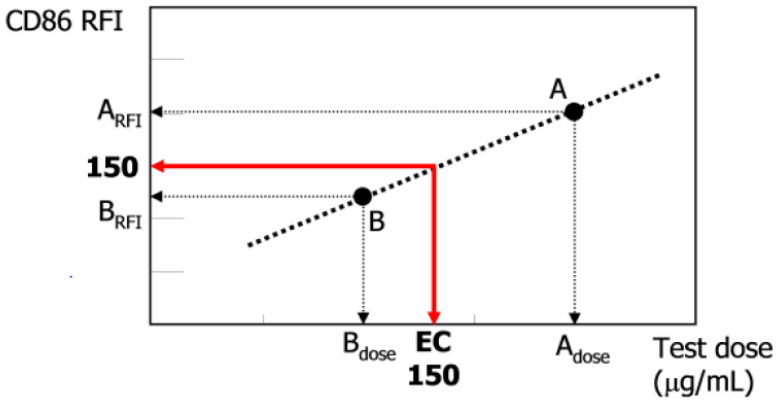
5.2.7 Data Analysis

If the RFI for any concentration exceeded the positive criteria (CD54 ≥ 200 and CD86 ≥ 150), the EC200 and EC150 were calculated by the following equation:

$EC200\ (CD54) = B_{dose} + [(200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})$
$EC150\ (CD86) = B_{dose} + [(150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})$

Where A_{dose}, B_{dose}, A_{RFI} and B_{RFI} were determined from the following chart (Figure 3):

Figure 3- Example dose response curve for CD86*



*ECVAM DB-ALM, *human Cell Line Activation Test (h-CLAT)*, DB-ALM Protocol No. 158. 2015: European Union Reference Laboratory for Alternatives to Animal Testing [28, 29].

If the EC200 or EC150 fell below the lowest dose, the values were extrapolated by the following equations.

$EC200\ (CD54) = 2\ exp[log2(B_{dose}) + (200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times [log2(A_{dose}) - Log2(B_{dose})]\}$
$EC150\ (CD86) = 2\ exp[log2(B_{dose}) + (150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times [log2(A_{dose}) - Log2(B_{dose})]\}$

Two independent runs were completed for MTNP and DHP as the data were consistent across both runs. A third run is necessary where the data are different between the first two runs, and a best-of-three decision is made regarding sensitization.

5.2.8 Criteria for a Valid Assay

For a test to be acceptable, the following criteria were met:

- Cell viability of medium and DMSO controls was more than 90 percent.
- RFI values for the DNCB control for both CD54 and CD86 exceeded the positive criteria (CD54 ≥ 200 and CD86 ≥ 150).
- RFI values for the DMSO solvent control did not exceed positive criteria.
- The MFI ratio of both CD54 and CD86 to isotype control for DMSO and media controls exceeded 105 percent.
- The cell viability of at least 4 doses was greater than 50 percent.

6 Results and Discussion

6.1 Reactivity Check

The THP-1 cells were checked and verified for reactivity to DNCB, $NiSO_4$ and lack of reactivity to LA. Cells reacted as expected, with DNCB and $NiSO_4$ eliciting positive reactions for both CD54 and CD86, while LA was negative in both (Table 2). The cells met criteria for further testing. Results for each flask are listed in the table.

Table 2: Results of Reactivity Check

Test article	Concentration (mg/mL)	Viability (% alive)	RFI (CD86)	RFI (CD54)	Positive (CD86/CD54)
Saline		91.07	100	100	N/N
		88.79	100	100	N/N
DMSO		89.14	100	100	N/N
		88.14	100	100	N/N
DNCB	0.0033	68.32	300.4	487.7	Y/Y
		67.99	379.0	334.0	Y/Y
	0.0040	63.64	178.6	711.5	Y/Y
		67.29	308.0	601.2	Y/Y
	0.0048	62.09	64.3	124.5	N/N*
		60.63	120.2	266.8	N/Y
$NiSO_4$	0.10	62.79	328.3	2995.7	Y/Y
		58.00	319.0	1811.1	Y/Y
Lactic Acid	1	89.02	79.0	145.2	N/N
		89.89	79.7	86.1	N/N

*****Based on the fact that lower treatment levels with DNCB were reactive, the mixed reactivity at the highest dose is not of concern.

6.2 Range finding Assay

Three independent dose finding assays were completed in order to determine the CV75 of MTNP in THP-1 cells. The average CV75 between the two assays which captured the CV75 was 0.0019 mg/mL (Table 3). Two independent assays were necessary to confirm that DHP was not cytotoxic at the highest test dose recommended by the protocol (1 mg/mL).

Table 3: Results of Range finding Assays

Compound	Assay	CV75 (mg/mL)	Average (mg/mL)
MTNP	Assay 1	0.0017	0.0019
	Assay 2	0.0022	
DHP	Assay 1	Undetermined	>1 mg/mL
	Assay 2	Undetermined	

6.3 CD54 and CD86 expression following compound exposure in THP-1 cells

Two independent tests were completed for MTNP for both CD54 and CD86. In all runs for both compounds, CD54 and CD86 were positive (Table 4). At the higher dosing levels, the RFI did not exceed positive criteria for MTNP, but this is commonly seen when cell viability is lower, even in the positive controls. At lower viability levels, there is a diffuse labeling of cytoplasmic structures which affects the background levels of stain and negates a positive response. The MTNP EC150 range for CD86 was 0.0001-0.0004 mg/mL and the EC200 range for CD54 was 0.0004-0.0007 mg/mL. DHP was not positive in any of the assays for either CD86 or CD54. Data are reported for the nominal concentrations of the compounds due to the fact that as of the writing of this report, concentration verification has not yet been completed by LS-MDV.

Table 4: Results of Compound Analysis

Compound	CD86 EC150 (mg/mL)	CD54 EC200 (mg/mL)	Positive Control (CD86/CD54)	Positive Test?
MTNP	0.0004	0.0006	Y/Y	Yes
	0.0001	0.0004	Y/Y	Yes
DHP	N/A	N/A	Y/Y	No
	0N/A	N/A	Y/Y	No

6.4 Criteria for Valid Assay

All criteria were met for all the assays.

7 Conclusions

As determined by h-CLAT, MTNP is considered positive by the test criteria, and DHP is negative. QSAR analysis by TOPKAT (BIOVIA, Inc.) predicted that both are potentially strong sensitizers. These data indicate that MTNP is most likely to be a skin sensitizer and DHP may be one, however the h-CLAT is one of several recommended skin sensitization tests, so results from further testing from these assays are recommended before definitive conclusions can be made.

8 Recommendations

MTNP appears to be a skin sensitizer upon analysis with the h-CLAT skin sensitizing assay when combined with QSAR analysis [6]. DHP is considered to be negative for skin sensitization by this assay, however, QSAR analysis indicates that it is a probable skin sensitizer. Further *in vitro* or *in*

vivo testing is recommended to more definitively determine the sensitizing potential of these compounds. The h-CLAT is one of many non-animal skin sensitizing tests, and it comprises part of an integrated testing strategy with two other *in vitro* approaches, the DPRA and the LuSens assay [7-11]. A comprehensive assessment of skin sensitization potential requires results from all three assays along with specific *in silico* analysis provides a more robust estimation of skin sensitization than h-CLAT alone [12]. As testing has only occurred with the h-CLAT, it is not yet possible to provide a definitive response as to the sensitization potential. Testing with the direct peptide reactivity assay will be conducted, and the LuSens test is currently under validation by APHC and should be available to complete the *in vitro* tests necessary for analysis. However, the robustness of the h-CLAT assay provides a strong indication that MTNP is a skin sensitizer and appropriate precautions should be taken when handling the material.

9 Point of Contact

Dr. Emily N. Reinke, the principal investigator, is the point of contact for this project. She may be reached at DSN 584-3980 or commercial 410-436-3980.

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Date

Appendix A

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Appendix B

QUALITY ASSURANCE STATEMENT

For: Toxicology Study No. S.0043494c-16, July 2017, Human Cell Line Activation Test of the Novel Energetics methyl trinitropyrazol (MTNP) and 1,3-dimethylhexahydropyrimidine (DHP), the following procedures were audited by the Quality Systems and Regulatory Compliance Office's Quality Assurance Unit:

Pre-Study Inspection

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Draft Type Protocol and Test Article Specific Modification GLP Review	01/10/2017	01/10/2017

In-life inspections

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
In-Vitro Skin Sensitization h-CLAT Assay Validation - PI qualifications and training	01/20/2017	01/24/2017
In-Vitro Skin Sensitization h-CLAT Assay Validation - General Requirements	01/20/2017	01/24/2017
In-Vitro Skin Sensitization h-CLAT Assay Validation - Maintenance and Cal of Equipment	01/20/2017	01/24/2017
In-Vitro Skin Sensitization h-CLAT Assay Validation - Labeling of Reagents	01/20/2017	01/24/2017
In-Vitro Skin Sensitization h-CLAT Assay Validation - Staining and FACS analysis	01/20/2017	01/24/2017
In-Vitro Skin Sensitization h-CLAT Assay - Reagents, Working Solutions and Cell Suspension Storage	02/16/2017	02/19/2017
In-Vitro Skin Sensitization h-CLAT Assay - Preparation of Stock and Working Solutions	02/16/2017	02/19/2017
In-Vitro Skin Sensitization h-CLAT Assay - Cell Suspension and Exposure	02/16/2017	02/19/2017

Post Study Inspection

Study Final Report and Raw Data GLP Review	08/30/2017	08/30/2017

Note 1: The above inspections were conducted to review the procedures for this type of study but may not have been for this specific study.

Note 2: All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table.

Note 3: In addition to the study specific critical phase inspections listed here, general facility and process based inspection not specifically related to this study are done monthly or annually in accordance with QA Standard Operating Procedure.

Note 4: This report has been audited by the Quality Assurance Unit (QSARC), and is considered to be an accurate account of the data generated and of the procedures followed

Michael P. Kefauver
GLP Quality Assurance Specialist, QSARC

6 NOVEMBER 2017
Date

Appendix C

Archives and Study Personnel

C-1. Archives

All raw data, documentation, records, protocols, contributing scientist reports, and a copy of the final report generated as a result of this study will be archived in the storage facilities of the Toxicology Directorate, APHC, for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

Records on the test system will be archived by the Toxicology Directorate for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

The present study used the Toxicology Study No. S.0043494c-16

The protocol, raw data, summary data, and the final report pertaining to this study will be physically maintained within Building E-2100, APHC. These data may be scanned to a computer disk. Scanned study files will be stored electronically with the study data in the archive.

Archived SOPs can be found in the Master Control database at APHC. Maintenance and calibration logbooks may be found in Room 1026, Building E-2100, APHC, APG, MD, 21010.

Archivist: Martha Thompson

C-2. Personnel

Management: Mark Johnson, Ph.D., D.A.B.T., Director, Toxicology; Michael J. Quinn, Ph.D., Program Manager, Health Effects Division (HEF)

Study Director: Emily N. Reinke, Biologist, HEF.

Quality Assurance: Michael P. Kefauver, Chemist, Quality Systems and Regulatory Compliance Office.

Appendix D

Reagents Used

Reagent	Supplier	Product Number	Lot Number	Expiration Date
THP-1	ATCC	TIB-202	62996831	N/A
RPMI-1640	Gibco	22400	1831722	09-17
FBS	Gibco	16140	1851521	11-21
2-Mercaptoethanol	Gibco	21985	1678663	02-18
Penicillin-Streptomycin	Gibco	15140	18535957	10-30-17
Saline	Sigma	S8776	RNBD7305	N/A
DMSO (TC)	Sigma	D2438	RNBD8224	06/17
Globulins	Sigma	G2388	017K7650V	N/A
BSA Fraction V	EMD Chemicals	12660	D00150383	N/A
D-PBS	GIbco	14190	1710578	06-18
Propidium Iodide	Sigma	P4864	MKBR1007V	N/A
CD54 Antibody, ICAM-1 Clone 6.5B5, FITC	Dako	F714301-8	20020092	08-18
CD86 Antibody, Hu Fun-1, FITC	BD	555657	50S1730	10-31-19
IgG1 (mouse), FITC	Dako	X092701-2	20023012	04-18
Flow Cytometer Beads	BD	650622	62638	09-17
Sheath Fluid	BD	342003	0000106241	3-12-18
2,4-dinitrochlorobenzene (DNCB)	Sigma	237329	BCBN7826V	N/A
Nickel Sulfate (NiSO4)	Sigma	656895	MKBT0269V	N/A
Lactic Acid (LA)	Sigma	W261106	MKBR4746V	N/A

Appendix E
Raw Data and Analysis

Experiment 1 – MTNP and DHP
Raw Data

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Dead Cells:All Events	10,000	***	***	100.00	1,754
Dead Cells:Live Cells	404	4.04	***	4.04	1,531
Live Cells:All Events	10,000	***	***	100.00	754
Live Cells:Live Cells	9,283	92.83	***	92.83	689
IgG DMSO:All Events	10,797	***	***	100.00	770
IgG DMSO:Live Cells	10,000	92.62	***	92.62	696
IgG DNCB:All Events	13,845	***	***	100.00	1,292
IgG DNCB:Live Cells	10,000	72.23	***	72.23	1,120
IgG DHP #1:All Events	11,222	***	***	100.00	893
IgG DHP #1:Live Cells	10,000	89.11	***	89.11	793
IgG DHP #2:All Events	10,849	***	***	100.00	793
IgG DHP #2:Live Cells	10,000	92.17	***	92.17	713
IgG DHP #3:All Events	11,001	***	***	100.00	842
IgG DHP #3:Live Cells	10,000	90.90	***	90.90	749
IgG DHP #4:All Events	10,957	***	***	100.00	847
IgG DHP #4:Live Cells	10,000	91.27	***	91.27	753
IgG DHP #5:All Events	10,999	***	***	100.00	857
IgG DHP #5:Live Cells	10,000	90.92	***	90.92	767
IgG DHP #6:All Events	10,960	***	***	100.00	826
IgG DHP #6:Live Cells	10,000	91.24	***	91.24	735
IgG DHP #7:All Events	11,355	***	***	100.00	880
IgG DHP #7:Live Cells	10,000	88.07	***	88.07	775
IgG DHP #8:All Events	11,125	***	***	100.00	865
IgG DHP #8:Live Cells	10,000	89.89	***	89.89	776
IgG MTNP #1:All Events	11,861	***	***	100.00	987
IgG MTNP #1:Live Cells	10,000	84.31	***	84.31	858
IgG MTNP #2:All Events	12,283	***	***	100.00	1,068
IgG MTNP #2:Live Cells	10,000	81.41	***	81.41	927
IgG MTNP #3:All Events	13,686	***	***	100.00	1,128
IgG MTNP #3:Live Cells	10,000	73.07	***	73.07	941
IgG MTNP #4:All Events	14,025	***	***	100.00	1,141
IgG MTNP #4:Live Cells	10,000	71.30	***	71.30	956
IgG MTNP #5:All Events	15,267	***	***	100.00	1,200
IgG MTNP #5:Live Cells	10,000	65.50	***	65.50	898
IgG MTNP #6:All Events	15,412	***	***	100.00	1,168
IgG MTNP #6:Live Cells	10,000	64.88	***	64.88	884
IgG MTNP #7:All Events	16,635	***	***	100.00	1,340
IgG MTNP #7:Live Cells	10,000	60.11	***	60.11	1,035
CD86 DMSO:All Events	10,973	***	***	100.00	1,843
CD86 DMSO:Live Cells	10,000	91.13	***	91.13	1,579
IgG MTNP #8:All Events	17,310	***	***	100.00	1,411
IgG MTNP #8:Live Cells	10,000	57.77	***	57.77	1,084
CD54 DMSO:All Events	10,742	***	***	100.00	955
CD54 DMSO:Live Cells	10,000	93.09	***	93.09	871
CD86 DNCB:All Events	14,779	***	***	100.00	4,755
CD86 DNCB:Live Cells	10,009	67.72	***	67.72	3,071
CD54 DNCB:All Events	14,631	***	***	100.00	2,738
CD54 DNCB:Live Cells	9,982	68.23	***	68.23	2,728
CD86 DHP #1:All Events	11,273	***	***	100.00	2,618
CD86 DHP #1:Live Cells	10,000	88.71	***	88.71	2,210
CD54 DHP #1:All Events	11,302	***	***	100.00	1,219
CD54 DHP #1:Live Cells	10,000	88.48	***	88.48	1,088
CD86 DHP #2:All Events	10,918	***	***	100.00	1,965
CD86 DHP #2:Live Cells	10,000	91.59	***	91.59	1,680
CD54 DHP #2:All Events	10,935	***	***	100.00	1,022
CD54 DHP #2:Live Cells	10,000	91.45	***	91.45	913
CD86 DHP #3:All Events	11,033	***	***	100.00	2,046
CD86 DHP #3:Live Cells	10,000	90.64	***	90.64	1,761
CD54 DHP #3:All Events	10,984	***	***	100.00	1,057
CD54 DHP #3:Live Cells	9,997	91.01	***	91.01	941
CD86 DHP #4:All Events	11,063	***	***	100.00	2,021
CD86 DHP #4:Live Cells	10,000	90.39	***	90.39	1,694
CD54 DHP #4:All Events	11,067	***	***	100.00	1,080
CD54 DHP #4:Live Cells	10,000	90.36	***	90.36	950
CD86 DHP #5:All Events	11,064	***	***	100.00	2,214
CD86 DHP #5:Live Cells	10,000	90.38	***	90.38	1,844
CD54 DHP #5:All Events	11,122	***	***	100.00	1,098
CD54 DHP #5:Live Cells	10,000	89.91	***	89.91	978
CD86 DHP #6:All Events	11,032	***	***	100.00	1,979
CD86 DHP #6:Live Cells	10,000	90.65	***	90.65	1,656
CD54 DHP #6:All Events	10,971	***	***	100.00	1,015
CD54 DHP #6:Live Cells	10,000	91.15	***	91.15	905
CD86 DHP #7:All Events	11,579	***	***	100.00	2,473
CD86 DHP #7:Live Cells	10,000	86.36	***	86.36	1,989
CD54 DHP #7:All Events	11,476	***	***	100.00	1,170
CD54 DHP #7:Live Cells	10,000	87.14	***	87.14	1,028
CD86 DHP #8:All Events	11,428	***	***	100.00	2,428
CD86 DHP #8:Live Cells	10,000	87.50	***	87.50	1,913
CD54 DHP #8:All Events	11,417	***	***	100.00	1,164
CD54 DHP #8:Live Cells	10,000	87.59	***	87.59	1,025

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
CD86 MTNP #1:All Events	12,312	***	***	100.00	3,610
CD86 MTNP #1:Live Cells	10,000	81.22	***	81.22	2,792
CD54 MTNP #1:All Events	12,214	***	***	100.00	1,409
CD54 MTNP #1:Live Cells	10,000	81.87	***	81.87	1,224
CD86 MTNP #2:All Events	12,709	***	***	100.00	4,130
CD86 MTNP #2:Live Cells	10,000	78.68	***	78.68	3,119
CD54 MTNP #2:All Events	12,938	***	***	100.00	1,579
CD54 MTNP #2:Live Cells	10,000	77.29	***	77.29	1,391
CD86 MTNP #3:All Events	14,827	***	***	100.00	5,411
CD86 MTNP #3:Live Cells	10,000	67.44	***	67.44	3,588
CD54 MTNP #3:All Events	14,590	***	***	100.00	2,040
CD54 MTNP #3:Live Cells	9,999	68.53	***	68.53	1,797
CD86 MTNP #4:All Events	14,677	***	***	100.00	5,217
CD86 MTNP #4:Live Cells	10,000	68.13	***	68.13	3,368
CD54 MTNP #4:All Events	14,531	***	***	100.00	2,061
CD54 MTNP #4:Live Cells	10,000	68.82	***	68.82	1,921
CD86 MTNP #5:All Events	15,765	***	***	100.00	5,278
CD86 MTNP #5:Live Cells	10,000	63.43	***	63.43	2,914
CD54 MTNP #5:All Events	15,664	***	***	100.00	2,041
CD54 MTNP #5:Live Cells	10,000	63.84	***	63.84	1,696
CD86 MTNP #6:All Events	16,774	***	***	100.00	5,825
CD86 MTNP #6:Live Cells	10,000	59.62	***	59.62	3,370
CD54 MTNP #6:All Events	7,431	***	***	100.00	1,892
CD54 MTNP #6:Live Cells	1,165	15.68	***	15.68	1,684
CD86 MTNP #7:All Events	10,632	***	***	100.00	3,873
CD86 MTNP #7:Live Cells	10,000	94.06	***	94.06	3,598
CD54 MTNP #7:All Events	17,610	***	***	100.00	1,716
CD54 MTNP #7:Live Cells	10,000	56.79	***	56.79	1,549
CD86 MTNP #8:All Events	28,388	***	***	100.00	6,243
CD86 MTNP #8:Live Cells	10,000	35.23	***	35.23	2,478
CD54 MTNP #8:All Events	20,812	***	***	100.00	1,738
CD54 MTNP #8:Live Cells	9,997	48.03	***	48.03	1,399

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Experiment 1
Data Analysis
2/15/2017

	Concentration (mg/mL)	Viability (%) (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
DMSO	0	92.62	696	1579	1.00	100.00		871	1	100	
DNCB Control	0.0040	72.23	1120	2738	1.83	183.24		2618	8.56	856	
DHP	0.2791	89.11	793	2210	1.60	160.48		1088	1.69	168.57	
	0.3349	92.17	713	1680	1.10	109.51		913	1.14	114.29	
	0.4019	90.9	749	1761	1.15	114.61		941	1.10	109.71	
	0.4823	91.27	753	1694	1.07	106.57		950	1.13	112.57	
	0.5787	90.92	767	1844	1.22	121.97		978	1.21	120.57	
	0.6944	91.24	735	1656	1.04	104.30		905	0.97	97.14	
	0.8333	88.07	775	1989	1.37	137.49		1028	1.45	144.57	
	1.0000	89.89	776	1913	1.29	128.77		1025	1.42	142.29	
MTNP	0.0006	84.31	858	2792	2.19	219.03		1224	2.09	209.14	
	0.0008	81.41	927	3119	2.48	248.24		1391	2.65	265.14	
	0.0009	73.07	941	3588	3.00	299.77		1797	4.89	489.14	
	0.0011	71.3	956	3368	2.73	273.16		1921	5.51	551.43	
	0.0013	65.5	898	2914	2.28	228.31		1696	4.56	456.00	
	0.0016	64.88	884	3370	2.82	281.54		1684	4.57	457.14	
	0.0019	60.11	1035	3598	2.90	290.26		1549	2.94	293.71	
	0.0023	57.77	1084	2478	1.58	157.87		1399	1.80	180.00	

Extrapolation Concentration (ug/mL)	MTNP EC150 RFI	Log2 Conc	Extrap.	ug/mL
0.64	219.03	-0.65	-1.27	0.41
0.76	248.24	-0.39		

Concentration (ug/mL)	EC200 RFI	Log2 Conc	Extrap.	ug/mL
0.64	209.14	-0.65	-0.70	0.62
0.76	265.14	-0.39		

Experiment 2 – DHP
Raw Data

Statistics

Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Geo Mean
MTNP #1 Viability Check:All Events	10,000	***	***	100.00	6,243
MTNP #1 Viability Check:Live Cells	2,760	27.60	***	27.60	532

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Dead:All Events	30,000	***	***	100.00	979
Dead:Live Cells	1,005	3.35	***	3.35	1,117
IgG DMSO:All Events	10,896	***	***	100.00	744
IgG DMSO:Live Cells	10,000	91.78	***	91.78	652
IgG DNCB:All Events	13,043	***	***	100.00	1,069
IgG DNCB:Live Cells	10,012	76.76	***	76.76	883
IgG DHP #1:All Events	11,030	***	***	100.00	761
IgG DHP #1:Live Cells	10,000	90.66	***	90.66	668
IgG DHP #2:All Events	11,090	***	***	100.00	768
IgG DHP #2:Live Cells	10,000	90.17	***	90.17	674
IgG DHP #3:All Events	11,073	***	***	100.00	759
IgG DHP #3:Live Cells	10,000	90.31	***	90.31	670
IgG DHP #4:All Events	11,234	***	***	100.00	794
IgG DHP #4:Live Cells	10,000	89.02	***	89.02	691
IgG DHP #5:All Events	11,191	***	***	100.00	787
IgG DHP #5:Live Cells	10,000	89.36	***	89.36	680
IgG DHP #6:All Events	11,651	***	***	100.00	885
IgG DHP #6:Live Cells	10,000	85.83	***	85.83	744
IgG DHP #7:All Events	11,334	***	***	100.00	815
IgG DHP #7:Live Cells	10,000	88.23	***	88.23	695
IgG DHP #8:All Events	11,254	***	***	100.00	800
IgG DHP #8:Live Cells	10,000	88.86	***	88.86	689
CD86 DMSO:All Events	11,056	***	***	100.00	1,697
CD86 DMSO:Live Cells	10,000	90.45	***	90.45	1,391
CD54 DMSO:All Events	11,051	***	***	100.00	1,072
CD54 DMSO:Live Cells	10,000	90.49	***	90.49	942
CD86 DNCB:All Events	12,935	***	***	100.00	4,509
CD86 DNCB:Live Cells	10,002	77.33	***	77.33	3,502
CD54 DNCB:All Events	13,268	***	***	100.00	2,452
CD54 DNCB:Live Cells	10,000	75.37	***	75.37	2,274
CD86 DHP #1:All Events	11,221	***	***	100.00	1,979
CD86 DHP #1:Live Cells	10,000	89.12	***	89.12	1,618
CD54 DHP #1:All Events	11,159	***	***	100.00	1,087
CD54 DHP #1:Live Cells	10,000	89.61	***	89.61	955
CD86 DHP #2:All Events	11,295	***	***	100.00	2,023
CD86 DHP #2:Live Cells	10,000	88.53	***	88.53	1,657
CD54 DHP #2:All Events	11,339	***	***	100.00	1,155
CD54 DHP #2:Live Cells	10,000	88.19	***	88.19	1,000
CD86 DHP #3:All Events	11,217	***	***	100.00	2,024
CD86 DHP #3:Live Cells	10,000	89.15	***	89.15	1,664
CD54 DHP #3:All Events	11,338	***	***	100.00	1,166
CD54 DHP #3:Live Cells	10,000	88.20	***	88.20	1,007
CD86 DHP #4:All Events	11,357	***	***	100.00	2,176
CD86 DHP #4:Live Cells	10,000	88.05	***	88.05	1,722
CD54 DHP #4:All Events	11,360	***	***	100.00	1,186
CD54 DHP #4:Live Cells	10,000	88.03	***	88.03	1,038
CD86 DHP #5:All Events	11,191	***	***	100.00	1,977
CD86 DHP #5:Live Cells	10,000	89.36	***	89.36	1,634
CD54 DHP #5:All Events	11,320	***	***	100.00	1,135
CD54 DHP #5:Live Cells	10,000	88.34	***	88.34	988
CD86 DHP #6:All Events	11,826	***	***	100.00	2,523
CD86 DHP #6:Live Cells	9,999	84.55	***	84.55	1,939
CD54 DHP #6:All Events	11,916	***	***	100.00	1,315
CD54 DHP #6:Live Cells	9,997	83.90	***	83.90	1,123
CD86 DHP #7:All Events	11,306	***	***	100.00	2,078
CD86 DHP #7:Live Cells	9,989	88.35	***	88.35	1,690
CD54 DHP #7:All Events	11,424	***	***	100.00	1,161
CD54 DHP #7:Live Cells	10,000	87.54	***	87.54	1,004
CD86 DHP #8:All Events	11,396	***	***	100.00	1,899
CD86 DHP #8:Live Cells	10,000	87.75	***	87.75	1,520
CD54 DHP #8:All Events	11,542	***	***	100.00	1,156
CD54 DHP #8:Live Cells	9,994	86.59	***	86.59	999

Experiment 2- DHP
Data Analysis

2/17/2017

	Concentration (mg/mL)	Viability (%)(IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
DMSO	0	91.78	652	1391	1	100		942	1	100	
DNCB Control	0.004	76.76	883	3502	3.54	354.40		2274	4.80	479.66	
DHP	0.28	90.66	668	1618	1.29	128.55		955	0.99	98.97	
	0.33	90.17	674	1657	1.33	133.02		1000	1.12	112.41	
	0.40	90.31	670	1664	1.35	134.51		1007	1.16	116.21	
	0.48	89.02	691	1722	1.40	139.51		1038	1.20	119.66	
	0.58	89.36	680	1634	1.29	129.09		988	1.06	106.21	
	0.69	85.83	744	1939	1.62	161.71		1123	1.31	130.69	
	0.83	88.23	695	1690	1.35	134.64		1004	1.07	106.55	
	1.00	88.86	689	1520	1.12	112.45		999	1.07	106.90	

Experiment 3- MTNP
Raw Data

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Dead Cells:All Events	10,000	***	***	100.00	1,736
Dead Cells:Live Cells	705	7.05	***	7.05	1,756
Live Cells:All Events	10,000	***	***	100.00	874
Live Cells:Live Cells	9,104	91.04	***	91.04	775
IgG DMSO:All Events	10,963	***	***	100.00	898
IgG DMSO:Live Cells	10,000	91.22	***	91.22	788
IgG DNCB:All Events	13,993	***	***	100.00	1,307
IgG DNCB:Live Cells	10,000	71.46	***	71.46	1,099
IgG MTNP #1:All Events	13,789	***	***	100.00	1,196
IgG MTNP #1:Live Cells	10,000	72.52	***	72.52	994
IgG MTNP #2:All Events	14,861	***	***	100.00	1,280
IgG MTNP #2:Live Cells	10,000	67.29	***	67.29	1,050
IgG MTNP #3:All Events	16,660	***	***	100.00	1,372
IgG MTNP #3:Live Cells	10,006	60.06	***	60.06	1,100
IgG MTNP #4:All Events	20,347	***	***	100.00	1,485
IgG MTNP #4:Live Cells	10,000	49.15	***	49.15	1,123
IgG MTNP #5:All Events	17,758	***	***	100.00	1,460
IgG MTNP #5:Live Cells	10,000	56.31	***	56.31	1,063
IgG MTNP #6:All Events	17,495	***	***	100.00	1,453
IgG MTNP #6:Live Cells	10,000	57.16	***	57.16	1,089
IgG MTNP #7:All Events	16,605	***	***	100.00	1,436
IgG MTNP #7:Live Cells	10,000	60.22	***	60.22	1,070
IgG MTNP #8:All Events	19,567	***	***	100.00	1,632
IgG MTNP #8:Live Cells	10,021	51.21	***	51.21	1,171
CD86 DMSO:All Events	11,081	***	***	100.00	2,124
CD86 DMSO:Live Cells	10,000	90.24	***	90.24	1,791
CD54 DMSO:All Events	11,487	***	***	100.00	1,199
CD54 DMSO:Live Cells	10,000	87.05	***	87.05	1,014
CD86 DNCB:All Events	14,685	***	***	100.00	5,862
CD86 DNCB:Live Cells	10,005	68.13	***	68.13	3,934
CD54 DNCB:All Events	15,304	***	***	100.00	2,912
CD54 DNCB:Live Cells	10,000	65.34	***	65.34	2,579
CD86 MTNP #1:All Events	14,129	***	***	100.00	6,338
CD86 MTNP #1:Live Cells	10,000	70.78	***	70.78	4,071
CD54 MTNP #1:All Events	15,045	***	***	100.00	2,407
CD54 MTNP #1:Live Cells	10,000	66.47	***	66.47	2,026
CD86 MTNP #2:All Events	16,116	***	***	100.00	6,965
CD86 MTNP #2:Live Cells	10,000	62.05	***	62.05	4,052
CD54 MTNP #2:All Events	16,654	***	***	100.00	2,663
CD54 MTNP #2:Live Cells	10,010	60.11	***	60.11	2,365
CD86 MTNP #3:All Events	17,867	***	***	100.00	7,863
CD86 MTNP #3:Live Cells	10,000	55.97	***	55.97	4,258
CD54 MTNP #3:All Events	18,295	***	***	100.00	3,107
CD54 MTNP #3:Live Cells	10,000	54.66	***	54.66	2,911
CD86 MTNP #4:All Events	21,474	***	***	100.00	8,897
CD86 MTNP #4:Live Cells	10,000	46.57	***	46.57	4,009
CD54 MTNP #4:All Events	21,980	***	***	100.00	3,253
CD54 MTNP #4:Live Cells	10,000	45.50	***	45.50	3,241
CD86 MTNP #5:All Events	18,501	***	***	100.00	7,055
CD86 MTNP #5:Live Cells	10,000	54.05	***	54.05	3,829
CD54 MTNP #5:All Events	18,966	***	***	100.00	2,485
CD54 MTNP #5:Live Cells	10,000	52.73	***	52.73	1,943
CD86 MTNP #6:All Events	17,745	***	***	100.00	6,161
CD86 MTNP #6:Live Cells	10,000	56.35	***	56.35	3,359
CD54 MTNP #6:All Events	18,052	***	***	100.00	2,136
CD54 MTNP #6:Live Cells	10,000	55.40	***	55.40	1,567
CD86 MTNP #7:All Events	20,616	***	***	100.00	6,981
CD86 MTNP #7:Live Cells	10,000	48.51	***	48.51	3,532
CD54 MTNP #7:All Events	18,260	***	***	100.00	2,134
CD54 MTNP #7:Live Cells	10,000	54.76	***	54.76	1,573
CD86 MTNP #8:All Events	22,866	***	***	100.00	6,953
CD86 MTNP #8:Live Cells	9,978	43.64	***	43.64	2,712
CD54 MTNP #8:All Events	23,505	***	***	100.00	2,273
CD54 MTNP #8:Live Cells	10,000	42.54	***	42.54	1,542

Experiment 3- TNBA
Data Analysis

2/28/2017

	Concentration (mg/mL)	Viability (%)(IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
DMSO	0	91.22	788	1791	1	100		1014	1	100	
DNCB Control	0.0040	71.46	1099	3934	2.83	282.65		2579	6.55	654.87	
MTNP	0.0006	72.52	994	4071	3.07	306.78		2026	4.57	456.64	
	0.0008	67.29	1050	4052	2.99	299.30		2365	5.82	581.86	
	0.0009	60.06	1100	4258	3.15	314.86		2911	8.01	801.33	
	0.0011	49.15	1123	4009	2.88	287.74		3241	9.37	937.17	
	0.0013	56.31	1063	3829	2.76	275.77		1943	3.89	389.38	
	0.0016	57.16	1089	3359	2.26	226.32		1597	2.25	224.78	
	0.0019	60.22	1070	3532	2.45	245.46		1573	2.23	222.57	
	0.0023	51.21	1171	2712	1.54	153.64		1542	1.64	164.16	

Extrapolation Concentration (ug/mL)	EC150 RFI	Log2 Conc	Extrap.	ug/mL
0.76	299.30	-0.39	-2.91	0.13
0.92	314.86	-0.13		

Extrapolation Concentration (ug/mL)	EC200 RFI	Log2 Conc	Extrap.	ug/mL
0.64	456.64	-0.65	-1.19	0.44
0.76	581.86	-0.39		